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The need for assessment of risks arising from interactions between NGT organisms from an EU perspective

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Abstract

Background New genomic techniques (NGTs) allow new genotypes and traits to be developed in different ways and with different outcomes compared to previous genetic engineering methods or conventional breeding (including non-targeted mutagenesis). EU GMO regulation requires an assessment of their direct and indirect effects that may be immediate, delayed or cumulative. Such effects may also result from the interactions of NGT organisms simultaneously present in a shared receiving environment or emerge from a combination of their traits. This review elaborates such potential interactions based on a literature review and reasoned scenarios to identify possible pathways to harm.

Main findings NGT organisms might be introduced into the environment and food chains on a large-scale, involving many traits, across a broad range of species and within short periods of time. Unavoidably, this would increase the likelihood that direct or indirect effects will occur through interactions between NGT organisms that are, for example simultaneously present within a shared environment. It has to be assumed that the cumulative effects of these NGT organisms may exceed the sum of risks identified in the distinct 'events'. Consequently, risk assessors and risk managers not only need to consider the risks associated with individual NGT organisms ('events'), but should also take account of risks resulting from their potential interactions and combinatorial effects. In addition, a prospective technology assessment could help the risk manager in defining criteria to minimize potential unintended interactions between NGT organisms through limiting the scale of releases.

Conclusions If genetically engineered (GE) organisms derived from NGTs are released into the environment, their potentially negative impacts need to be minimized. As with all GE organisms, it is, therefore, crucial to not only assess the risks of the individual events, but also their potential interactions which can trigger direct and indirect effects with adverse impacts. It is necessary to develop hypotheses and specific scenarios to explore interactions between NGT organisms and possible pathways to harm from the perspective of the precautionary principle. In addition, the introduction prospective technology assessment could provide an instrument for the risk manager to control the scale of releases of NGT organisms.

Keywords New genomic techniques, Genetically engineered organisms, Genome editing, GMO regulation, Risk assessment, Direct and indirect effects, Interactions, Cumulative long-term effects, Accumulated risks, Prospective technology assessment

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Background

According to EU GMO legislation [1], inter alia, genetically engineered (GE) organisms (or 'genetically modified organisms', GMOs) derived from "recombinant nucleic acid techniques" [Annex 1A, Part 1 of 1] fall within the scope of the regulation. As clarified by the EU Court of Justice (Case C-528/16), this also applies to organisms derived from 'new genomic techniques' (NGT).

As a general principle, the EU requires each individual genetically engineered organism ('event') to undergo risk assessment on a case-by-case basis before it is released into the environment or placed onto the market. The detailed risk assessment requirements are set out in Annex II of Directive 2001/18/EC [1] which was amended by Commission Directive (EU) 2018/350 [2]. It foresees the risk assessment of each event, taking into account its intended and unintended effects since "intended and unintended changes can have either direct or indirect, and either immediate or delayed effects on human health and on the environment." [Annex (C1) of 2]. Therefore, as introduced in Annex (C1) by Commission Directive (EU) 2018/350 [2], the risk assessment "shall identify the intended and unintended changes resulting from the genetic modification and shall evaluate their potential to cause adverse effects on human health and on the environment."

In the context of Commission Directive (EU) 2018/350, risk assessment cannot be confined to the individual event only, it also needs to take interactions involving other GE organisms into account. This is further underlined in Recital 19 of the Directive 2001/18/EC [1]: "A case-by-case environmental risk assessment should always be carried out prior to a release. It should also take due account of potential cumulative long-term effects associated with the interaction with other GMOs and the environment." Furthermore, as defined in the amended Annex (C3) of Commission Directive (EU) 2018/350 [2], "adverse effects may occur directly or indirectly through exposure pathways or other mechanisms which may include: (...) interactions with other organisms (...)". In conclusion, risk assessment must also include potential interactions between GE organisms which are released into a shared receiving environment and potential combinations of their traits.

EU GMO regulation contains further specific provisions relating to interactions between GE organisms or a combination of their traits in risk assessment. For example, Annex II of Directive 2001/18/EC [1] in its "Principles for the environmental risk assessment" gives weight to cumulative long-term effects: "A general principle for environmental risk assessment is also that an analysis of the cumulative long-term effects relevant to the release and the placing on the market is to be carried out.

'Cumulative long-term effects' refers to the accumulated effects of consents on human health and the environment (...)." Furthermore, similarly to Commission Directive (EU) 2018/350 [2], Commission Implementing Regulation (EU) No 503/2013 [3] also requires the assessment of stacked events in regard to their "potential additive, synergistic or antagonistic effects resulting from the combination of the transformation events."

The European Food Safety Authority (EFSA) in its "guidance on the environmental risk assessment of genetically modified plants" [4] considers effects resulting from interactions between genetically engineered plants, such as those causing indirect effects on management and production systems. The risks of long-term cumulative effects and stacked events are also taken into account. However, one example not specifically mentioned concerns interactions between GE organisms within a shared receiving environment that are released within a short period of time and may, therefore, not be considered to be long-term cumulative.

For the purpose of this review, there are several categories which have to be distinguished by using specific terminology: (1) EU GMO regulation refers to GMOs which have to undergo mandatory approval processes and other GMOs which are exempt from these approval processes, e.g., plants derived from physical and chemical mutagenesis. The wording 'genetic engineering' (GE) is used throughout the publication as a synonym for those GMOs which have to undergo mandatory approval processes. (2) The wording 'established genomic techniques' (EGTs) is used to distinguish older transgenic plants (derived from non-targeted insertions of transgenes) from those more recently generated using NGTs [see also 5]. It is important to understand that both these categories (EGT and NGT) refer to GMOs which have to undergo mandatory approval processes (GE).

A soaring number of NGT applications across many traits and species may soon be released into the environment within a short period of time [see, for example, 6]. Against this backdrop, and in light of existing evidence regarding the impact of large-scale cultivation on the environment, the purpose of this review is to highlight the need for assessment of environmental risks arising from interactions between NGT organisms. We started from horizon scanning of studies on the environmental risk assessment of transgenic plants reporting unintended effects from cultivation, including interactions between different events and their traits. Further research was conducted on examples to explore the potential interactions of NGT organisms that may be released into a shared environment. Databases that provide a comprehensive overview of NGT applications [such as 6, 7] were used to select examples for relevant

organisms. Problem formulation is a crucial step in risk assessment. Therefore, we developed plausible risk scenarios to examine such potential interactions. Since the issue of potential interactions between NGT organisms is not yet well reflected in scientific literature, most of our analysis had to be done 'from scratch.' Therefore, systematic and continuous horizon scanning was the most crucial aspect in terms of our methodology, followed by targeted analyses of potentially relevant publications.

Our review provides a first, albeit non-comprehensive overview of findings in regard to interactions between GE organisms, in particular between NGT organisms that may cause direct or indirect effects. In this context, we consider interactions such as enabled by simultaneous presence of several NGT events belonging to the same species or to different species within a shared receiving environment. These interactions may be additive, synergistic, antagonistic or cumulative.

As shown in the review, there are good reasons why interactions between NGT organisms deserve the particular attention of the risk assessor and the risk manager: plants developed with NGTs may include many more different traits and species compared to transgenic plants approved for the market so far. In addition, NGT applications may be relevant to all domains of life, including non-domesticated species [see, for example, 8]. If these assumptions are correct, NGT organisms might be introduced into the environment on a large-scale in terms of numbers and traits, across a broad range of species and within short periods of time. Unavoidably, this would also increase the likelihood that direct or indirect effects will, for example, occur through interactions between those organisms. Therefore, their intended and unintended interactions and potentially emerging hazards may need more attention and consideration compared to EGT organisms.

Specific technical characteristics of NGTs

NGTs, also known as genome editing or new genetic engineering, allow new genotypes and traits to be generated in different ways and with different outcomes compared to previously used genetic engineering methods or conventional breeding (including non-targeted mutagenesis) [9–11].

So-called site directed nucleases (SDN), such as CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR associated) [12], are highly relevant in this context: they can be designed to target specific DNA sequences in the genome to knock out gene functions (i.e., SDN-1), to induce repair mechanisms for specific alterations of particular nucleotides (i.e., SDN-2) or whole genes (i.e., SDN-3). Depending on whether a repair template is used or not, these methods

can induce either non-specific changes (SDN-1) via non-homologous end joining (NHEJ) repair mechanisms or induce specific changes to nucleotide sequences (SDN-2 or SDN-3) via homologous recombination mediated by homology directed repair (HDR). The induced changes at or around the target site can be substitutions, deletions or insertions of one or more base pairs. Depending on the specific SDN-1 or SDN-2 application, more extensive overall changes are possible. For example, multiplexing can target several genes simultaneously in a single application [13–15]. Repeated applications of SDN-1 or SDN-2 can also be combined [16]. Changes involving the insertion of whole (cis- or trans-) genes (including gene-stacking) are also possible (SDN-3) and are mediated by the use of specific donor DNA [9, 17]. For this review, we mostly focused on applications using CRISPR/Cas which is currently the most important tool for developing NGT organisms [8]. Other nucleases, such as TALENs (transcription activator-like effector nucleases) or variations of CRISPR nucleases [see, for example, 8], are also relevant, but so far of less importance for NGT applications in plants and animals.

The following section describes some specific characteristics relevant to NGT applications using CRISPR/Cas and also illustrates their technical potential and associated risks by giving special weight to the perspective of the precautionary principle:

a) Greater precision

In comparison to EGTs, the NGTs can be used to introduce genetic changes with much greater precision. Typically, SDNs can be used to directly target sites [12, 18, 19], whereas previous transformation processes introduce additional DNA sequences at random sites [see, for example, 20–22]. However, NGTs are based on processes involving several technical steps that, in the case of plants, very often include transformation processes, e.g., biolistic methods or the use of *Agrobacterium tumefaciens*, which are also used in EGTs. These non-targeted methods are used to introduce the nucleases into the cells [16] and may lead to unintended effects in off-target regions [for example 23, 24]. As pointed out in several publications, there are additional reasons why higher precision still appears to be challenging and should be improved [for overview, see 9, 16, 25–27].

In comparison to conventional breeding in plants which also uses non-targeted mutagenesis, the overall number of mutations is typically lower in NGT plants [25]. However, these comparisons need to be put into context: conventional breeding methods depend on high genetic diversity that can be used for crossing and selection. Thus, the higher number of mutations caused by non-targeted mutagenesis is generally desired while, at

the same time, some of the mutations are considered to be disadvantageous [28].

Furthermore, the specific sites of the mutations and the (intended or unintended) resulting gene combinations from the NGTs may, in many cases, be unlikely to occur with conventional methods (see below). These genetic changes may thus cause unintended direct or indirect effects within the complex networks of genes, proteins and other biologically active molecules not so far observed (see also below). Some of these unintended metabolic and physiological effects can also be considered to be ‘trade-offs’ which can still emerge in cases where the genetic intervention is targeted and precise (see below).

b) Overcoming the limitations of natural genome organization

NGTs can be used to achieve genomic changes extending beyond what is known from conventional breeding, even without the insertion of additional genes. If compared to methods of conventional breeding (including non-targeted mutagenesis), NGTs can overcome the boundaries of natural genome organization. Relevant factors include repair mechanisms, gene duplications, genetic linkages and further epigenetic mechanisms [see, for example, 29–37]. By overcoming these boundaries, NGTs can make the genome much more extensively available for genetic changes [10, 16]. The resulting genotypes (the patterns of genetic changes) can be vastly different compared to those derived from conventional breeding, both in regard to intended and unintended changes [38, 39] even though there may still be some limitations to the effectiveness of the nucleases [40]. This means that it is possible to generate genotypes which are highly unlikely to result from natural processes or traditional breeding techniques. As a result, more ‘extreme’ biological characteristics can be achieved with NGTs in comparison to conventional breeding methods. These can, however, also be associated with more significant ‘trade-offs’ in comparison to conventional breeding [41–45].

c) Changes in the allelic diversity of populations within short periods of time

For example, Barbour et al. [46] showed that a higher allelic diversity in plants has an impact on different species within an experimental food web. This may also play a crucial role in the stability of ecosystems and food webs. CRISPR/Cas applications can, in particular, be used to make gene variants within a population more uniform, i.e., the frequency of the abundance of different allelic variants can be reduced, the alleles can be changed or the respective gene (-family) can be blocked in its functions.

In general, in regard to allelic uniformity, CRISPR/Cas applications are very much more efficient than conventional breeding methods, including the knocking out of many or all copies of a gene family in parallel using SDN-1 processes [47]. Therefore, if there are large-scale releases of NGT organisms into the environment in short periods of time, their impact on genetic diversity and associated ecosystems can extend far beyond what might be expected compared to natural processes and conventional breeding techniques.

d) Pervasive changes even without the insertion of additional genes

Even without the insertion of additional genes, changes in genotypes and phenotypes can be pervasive and involve major changes in gene expression, metabolism in the cells and characteristics of the organisms [see, for example, 47]. Changes can be brought about by, for example, knocking out most or all copies of a gene family, changing several different genes in parallel (multiplexing), or altering elements responsible for gene regulation [13–16]. Such technical interventions can, lead to major and unprecedented changes in plant composition, resulting in traits without a ‘history of safe use’ also be associated with unintended effects [38, 39, 41, 47–49]. As the case of de novo domesticated tomatoes [47] examined by EFSA [48] shows, the NGT process results in a combination of known gene variants with a genomic background of *S. pimpinellifolium*, thus resulting in ‘new’ tomatoes without known comparators.

e) Wide range of species and applications

The range of species accessible to NGTs extends far beyond applications of previously used genetic engineering techniques (EGTs). While their effectiveness may differ from case to case (especially in regard to SDN-2 and SDN-3 processes), these applications include a wide range of food plant species and livestock, and also non-domesticated species including trees and other plants, insects, vertebrates and microorganisms, thus extending across all domains of life [overview in: 5, 38, 39]. Several specific NGT applications are designed for use in wild populations, including gene drives (SDN-3) [52, 53] and the intended release of genetically engineered viruses, also including so-called Horizontal Environmental Genetic Alteration Agents (HEGAA) [54, 55]. Many of the species targeted by NGT applications also have the potential to persist and spread over longer periods of time without effective control. This may give rise to next-generation effects which were not observed in the laboratory [56].

f) Complex interactions—also triggered by simultaneous presence in the environment

Large numbers of GE organisms derived from NGTs, including various species with a wide range of different characteristics (intended or unintended), could be released into the same receiving environment within a short period of time [see, for example, 8]. Depending on the scale of the releases, their duration and the characteristics of the organisms, these NGT organisms may also intentionally or unintentionally interact with each other. A number of applications are already designed for complex interactions after release into the receiving environment, e.g., changes in the microbiome of the soil [57–59], in plants [60–63], in insects [64–72] or in corals [73]. Moreover, some of the applications use a technique known as ‘paratransgenesis’ which aims to change the biological characteristics of the host by genetically engineering its microbiome [74].

In summary, sections (a–f) listed above, all illustrate the novelty of NGTs that allows the development of new genotypes and traits in different ways and with different outcomes than could have been expected with earlier GE methods or conventional breeding (including non-targeted mutagenesis).

Scenarios for interactions between NGT organisms that are relevant to risk assessment

Given the specific characteristics listed above, both direct and indirect effects resulting from interactions between NGT organisms may have an extensive impact on ecosystems. This is especially relevant to large-scale releases of NGT organisms which may comprise a broad range of species and traits. Such large-scale releases may be driven by expectations of hypothetical benefits from NGT organisms [75], and at the same time, lead to high- and long-term environmental exposure to NGT organisms. Large-scale releases undoubtedly increase the likelihood of direct or indirect effects from interactions between the NGT organisms. Therefore, the need to risk assess the interactions between NGT organisms may be much more crucial in comparison to previous applications of genetic engineering [see also 76]. In this context, the development of risk scenarios can help to generate plausible hypotheses (problem formulation) and guide the steps in risk assessment [see 4]. The following section contains examples of possible relevant scenarios:

Scenario 1: interactions resulting from releases of NGT events involving different traits and several species

The first hypothesis, we suggest should be tested, is: ‘Releases of different NGT events into a shared receiving environment may lead to a scaling up of adverse impacts due to interactions between the NGT organisms.

There may be a tipping point for such releases which could trigger irreversible damage caused by exposure to effects unlikely to result from natural processes [77]. This hypothesis is based on the following specific characteristics of NGTs as described above: (i) the differences between the genotypes and phenotypes of NGT organisms compared to those which can be expected to evolve naturally or are derived from conventional breeding (see characteristics b, c and d); (ii) the broad range of species across all domains of life, with a possibly significant number of the NGT organisms having the potential to persist and spread without effective control (see characteristics e); (iii) the technical potential of NGTs allowing the development of various GE organisms with a wide range of different characteristics (intended or unintended) within a short period of time, all of which may be released into a shared receiving environment (characteristics a and f). The potential interactions between NGT organisms may be additive, synergistic, antagonistic or cumulative, and may cause direct or indirect effects with immediate or delayed adverse environmental impacts.

Camelina sativa with changes in oil content [78] could be the starting point for testing this hypothesis. It is an example which can be used to exemplify several of the technical characteristics of NGT plants as mentioned [38]: both, its genotype and phenotype go beyond what was achieved using conventional breeding, thus causing ‘extreme’ variants of its biological characteristics (for more details see below, Scenario 2). Furthermore, this species has the potential to persist, propagate and spread uncontrollably in the environment. The biological characteristics resulting from the genetic intervention may cause a change in interactions with many species within the receiving environment: as shown by Kawall, 2021 [38], this may affect pollinators, pest insects and other wild life species feeding on the plants. However, we are not (yet) aware of enough data from other NGT organisms belonging to other species that may share the same environment to establish a specific scenario as outlined in the first hypothesis. We, therefore, refrained from presenting a specific example for Scenario 1 and instead introduced it as an example in Scenario 2.

A summarized reasoning of our first hypothesis may read as follows: the intended and/or unintended effects resulting from NGT applications mean that the release of the NGT organisms may not be ‘neutral’ to the ecosystems. Disruptive, i.e., adverse effects, are more likely or less likely depending on their biological characteristics (intended or unintended), environmental exposure (as caused by the number of organisms, traits and events released, their distribution and persistence) and their potential interactions. Even if distinct individual

events are considered to be 'safe,' uncertainties or even unknowns will emerge from their interactions with other NGT organisms released into the same environment. Therefore, environmental risk assessment of NGT organisms should take their interactions into account, as these may cause adverse environmental effects. Potential adverse effects that should be included in Scenario 1 comprise damage to biodiversity, breakdown in food production or disruption of ecosystem services.

This first hypothesis is mostly set out in general terms and describes numerous impact factors and potential interactions between GE organisms. The development of a more specific hypothesis would be needed in order to draw conclusions for regulatory decision-making. We have, therefore, developed a more specific second scenario focusing on different NGT traits, generated within the same species.

Scenario 2: interactions emerging from releases of several NGT events from one species but with different traits

The second hypothesis that we suggest should be tested, is: 'The unintended effects of different traits introduced into the genome of one species if accumulated by simultaneous cultivation and/or by further stacking and/or spontaneous crossings of the events, may lead to a scaling up of hazards due to unintended interactions.' In this scenario, we have chosen NGT applications in plants of the same species to discuss interactions between their traits (and the respective events) that may trigger direct or indirect effects that are associated with hazards. In this context, the overall impact may also depend on the susceptibility of the species to a range of common pathogens and their potential for gene flow, crossings and the degree of intended stackings. We, therefore, selected two species used in several NGT applications (SDN-1): *Camelina sativa* and *Triticum aestivum*. One of the reasons for choosing *Camelina sativa* are the biological characteristics of this species, which is able to persist and spread in the environment. It can also spontaneously cross with other varieties of the same species and other wild relative species [38]. *Triticum aestivum* was chosen because of the many NGT applications that offer a wide range of different traits [79].

Example 1: In *Camelina sativa*, NGT applications are primarily used to change the quantity and quality of oil [80]. The aim in most of the plants being developed is to lower the percentage of polyunsaturated fatty acids (PUFA). These fatty acids (PUFA) are generally considered to be desirable and beneficial for healthy food. However, for use as agrofuel, the aim is to increase the oxidative stability of the oil by reducing the content of PUFA. At the same time, the intention is also to increase the quantity of oil. Several metabolic pathways can be

used to impact the oil content of the plants by changing the genotype at different sites [80]. *Camelina sativa* is an allohexaploid species, which means that many alleles are present in several copies. As explained above, NGTs have the novel potential to overcome this constraint by changing all copies of a gene at the same time (technical characteristics b & d). Recent attempts at CRISPR/Cas9-induced gene mutagenesis in camelina have been successful [78, 81, 82], but have also resulted in negative effects, e.g., smaller growth and a reduced number of seeds, due to the pervasive changes in the metabolism of fatty acids [45].

In regard to scenario 2, it is crucial that NGT camelina can persist, propagate and spontaneously cross. This means that new combinations of NGT traits may occur and persist unnoticed. In some cases, the resulting plants may suffer from low fitness [45] and therefore are not likely to persist in the environment for longer periods of time. However, the biological characteristics associated with a reduced content of PUFA in the plants suggest a more complex landscape: as shown by Kawall [38] in *Camelina sativa* with changed oil composition [78], the genetic intervention may trigger direct and indirect effects and impact various other functions, e.g., plant defense mechanisms, interactions with pollinators or associated food webs. In worst case scenarios, some of these characteristics may be associated with enhanced spread and a severe impact on ecosystems: for example, spontaneous crossings of NGT camelina may, unnoticed and with no intention, result in plant compositions that negatively impact the health of wild species feeding on the plants. In these circumstances, some of these wild species may suffer from a population decrease, whereas the plants might gain an advantage due to reduced losses from herbivores. This may lower the chances of controlling the spread of the plants in the environment, and thus severely impact both natural plant populations of species able to cross with the NGT plants and populations of animal species feeding on the plants.

Example 2: the combination of traits may also play a role in other species being developed for various NGT applications, e.g., *Triticum aestivum*. We are currently aware of more than 20 NGT projects focusing on bread wheat (*Triticum aestivum*) [79]. To exemplify our scenarios, we selected three traits under development (see below). The traits were introduced by using NGTs (SDN-1) and other multistep processes (involving previously used genetic engineering methods such as biolistic methods). *Triticum aestivum* has a huge genome, comprising six sets of chromosomes [83] which is a severe constraint in conventional wheat breeding since, in many cases, a high number of gene duplications are involved in a specific trait. As explained above, NGTs have the

novel potential to overcome this constraint by changing all copies of a gene at the same time (technical characteristics b & d). As far as the selected NGT traits (see below) are concerned, there are reasons to assume that the intended (on-target) genetic alterations and resulting more ‘extreme’ traits are linked to ‘trade-offs’ as well as unintended biological characteristics.

Trait 1, reduction in gluten: Gluten proteins in wheat are thought to trigger several gluten-related disorders, including celiac disease [84]. It is known that alpha-gliadin peptides contribute to the overall concentration of gluten in bakery products [79]. Their genes occur within a large family of genes that are present in multiple copies at different locations in the genome. With the help of the CRISPR/Cas nuclease, scientists succeeded in 2018 in switching many of them off: 35 out of the 45 genes necessary to produce alpha-gliadins were knocked out [49]. This resulted in a new wheat genotype that is highly complex in regard to risk assessment [11]. As EFSA, in 2021 [11] states: “(...) *the large number of mutations required to achieve gluten-free wheat is far beyond any plant previously assessed. This is likely to require SynBio approaches to correctly identify all gliadins and glutenins in the hexaploid genome of bread wheat and to identify an engineering strategy that introduced mutations of the correct nature and positions in each gene to prevent the accumulation of any peptide fragments associated with initiation of the inflammatory cascade*”. Regardless, the application of CRISPR/Cas resulted in biological characteristics that go beyond what can be achieved with conventional breeding. This ‘extreme’ trait may be associated with specific ‘trade-offs’: gliadins are, for example, known to play an important role in the response of the plants to stress conditions, including drought and heat [85–87]. Therefore, large reductions in the content of alpha-gliadins may also unintentionally impact the heat and/or drought tolerance of this new genotype.

Trait 2, reduction in acrylamide: CRISPR/Cas9 was used to reduce the content of the free amino acid, asparagine, in wheat [42]. Free asparagine is present in higher concentrations in wheat grain. It is a precursor for acrylamide, which forms during the baking, toasting and high-temperature processing of foods made from wheat. Acrylamide has been shown to be carcinogenic. The gene (*asn2*) targeted in this case, occurs a total of six times in the wheat genome. In some of the NGT wheat plants developed by Raffan et al. [42], the asparagine content in wheat grain was reduced by 90% compared to the wild type, something which has not previously been achieved using other methods. This ‘extreme’ trait is associated with ‘trade-offs’: while the gene function involved in production of the amino acid asparagine was successfully blocked, this also creates

other problems since asparagine is involved in seed germination, plant growth, stress response and defense mechanisms. It was found that some lines of this CRISPR-wheat almost lost their capacity to germinate [42]. There is as yet no published data on the resistance of these NGT plants to biotic and abiotic stressors.

Trait 3, reduction in susceptibility to powdery mildew: the mildew resistance locus (*mlo*) gene in barley is of interest in several projects. There are three different *mlo* genes involved in resistance to powdery mildew found in natural populations. In one study, the *mlo* gene was targeted in hexaploid wheat by TALENs [88]. The nuclease introduced alterations in all three homoeoalleles of *mlo* in wheat, enabling their parallel knock-out. This has never previously been achieved using non-targeted mutagenesis or other breeding methods [see 38]. The simultaneous knock-out of the three homoeoalleles conferred a broad-spectrum resistance to powdery mildew in the new wheat lines. However, some unintended effects were described in these lines (i.e., leaf chlorosis under growth conditions), which were not observed to the same degree in randomly mutated plants [89]. Growth aberration, accelerated senescence, induced necrosis, increased susceptibility to other fungal pathogens are unintended effects described in the context of this trait. To a certain extent these ‘trade-offs’ are also known from conventional breeding and may be overcome only by further research [43].

Some of the interactions and combinatorial effects, such as reduced fitness of crop plants or lowered germination rate in seeds, might easily be detected by the breeders. Others, such as impacts on ecosystems and food web might be overlooked and remain undetected for a longer period of time. Effects that may occur from spontaneous crossings are a particular challenge in risk assessment and risk management. As mentioned, spontaneous crossings would be expected in species like camelina, but not necessarily in wheat. However, research is underway to develop new varieties by using specific lines of wheat which show a much higher potential for spontaneous crossings [90]. The idea behind this research is to ease crossings from wild relative species with desirable characteristics by enhancing the likelihood of crossover between related (homoeologous) chromosomes. If such varieties are grown in the fields in future, they may also increase the rate of spontaneous crossing with NGT lines.

To sum up the examples of Scenario 2, we would like to highlight preliminary observations regarding (i) the susceptibility of the overall plant population to stressors if varieties with different traits are cultivated together; (ii) genomic effects that may occur from further crossings

and (iii) accumulation of unintended genetic changes due to the technical processes of NGTs.

(i) As mentioned, the susceptibility of the overall plant population to biotic and abiotic stressors may be affected if NGT plant varieties with different traits are cultivated together. One reason for this assumption is a common range of pathogens which may affect plant health. Increased susceptibility to plant diseases of a variety with one trait may also cause enhanced pest pressure and damage in varieties with other traits. For example, if varieties with traits of (unintended) higher susceptibility to biotic stressors are grown together with varieties with traits of (unintended) reduced tolerance to abiotic stressors, this may cause the collapse of plant populations which would otherwise have been successfully cultivated.

(ii) Furthermore, different traits can be stacked by technical means or conventional breeding or spontaneous crossings, and thus result in offspring with biological characteristics which were absent in the parental events. This may, for example, lead to plant compositions which negatively impact the ecosystems, the food webs, the health of wild species and consumers. In general, if traits do not have a history of conventional breeding, the effects resulting from their interactions and combinations may be hard to predict [see 56]. Even if each of the events themselves are considered to be 'safe' in risk assessment, their offspring may show next-generation effects associated with unexpected risks caused by genomic interactions. Such genomic interactions may, for example, be caused by epistasis: current research on NGT applications in tomatoes shows that the effects of cryptic gene variants may depend on their genetic background [see, for example, 70–72]. Cryptic variations are considered to be mutations that, regardless of whether they occur naturally or are introduced by technical processes, have little or no phenotypic consequences unless exposed to additional genetic or environmental interactions. These cryptic variations may then lead to unpredictable and sometimes detrimental outcomes due to epistatic interactions with other genetic variations [91, 92, 94]. These findings are also relevant to other plant species, such as wheat [95–97]. Therefore, the genomic interactions emerging from gene flow, from conventional crossings and technical stacking are relevant to the assessment of intended and unintended genetic changes caused by the processes of NGT.

(iii) Finally, further crossings may also lead to accumulations of unintended off-target genetic alterations, caused by the multistep processes of NGT (see technical characteristics) involving for example *Agrobacterium tumefaciens* or biolistic methods to generate the above-described NGTs traits [42, 49, 78, 88]. They are known for causing specific unintended effects in EGTs [20–23]

and NGTs [24, 26, see also 98]. However, so far, in many cases of NGT applications as well as in the context of the examples used in Scenario 2, no comprehensive data on potential off-target effects have been published. In future, specific attention should be paid to the assessment of unintended genetic effects caused by the multistep processes inherent to NGTs, since they are also of relevance to the assessment of potential interactions both between and combinations of NGT organisms.

A summarized reasoning of our second hypothesis may read as follows: If NGTs are used to generate different traits in one species, the resulting intended and/or unintended genetic changes may lead to interactions between the NGT organisms, and thus result in effects that are relevant to risk assessment. Simultaneous cultivation, further crossings and technical stacking of the various events need to be taken into account. The resulting effects may be dependent on specific combinations of intended or unintended genetic variants, the intended traits and/or the exposure to stress conditions in the receiving environment. Even if all individual events were considered to be 'safe', uncertainties or unknowns will still remain due to interactions of the intended and unintended genetic changes and associated effects in each event. Therefore, the environmental risk assessment of individual events is not sufficient to predict and assess all these interactions. Potential adverse effects may impact plant health and their response to biotic and abiotic stressors, causing a breakdown in food production or disruption in agro-ecological systems. Additional direct or indirect effects (not discussed in here) may emerge in the context of food safety if the harvest of the different traits is mixed into diets.

Some experience from transgenic plants

So far only a few traits (mainly herbicide tolerance and insecticidal toxicity) have been established in transgenic plants. Nevertheless, several unintended interactions between those plants have already been observed and discussed, and are associated with environmental risks. For example, potential disruptive effects on ecosystems have been described by Vázquez-Barríos et al. [99] in regard to glyphosate-resistant transgenic cotton that produces insecticidal Bt toxins. These transgenic cotton plants have successfully invaded and outcrossed into natural cotton populations in Mexico, which is one of the centers of diversity for wild cotton *Gossypium hirsutum* [100]. The authors reported that the expression of both transgenic traits in wild cotton under natural conditions changed extra-floral nectar production, and thus changed its association with different ant species. Vázquez-Barríos et al. [99] discuss to which extent the genomic and metabolic interactions of the combined traits promote

the spread of the transgenic cotton. Further research is needed to identify the underlying causes, for example, by comparing in more detail the single traits with the combined traits occurring in spontaneous crossings.

In Brazil, populations of white flies (*Bemisia tabaci*) are increasing in fields where insecticidal transgenic soybeans are grown. Originally, the rise in the number of white flies was explained by a reduction in insecticide sprayings. However, laboratory experiments revealed other findings [101]: the white flies suck fluid from the plants and appear to benefit from specific biological characteristics of the transgenic soybeans. This finding seems to be especially true for transgenic soybeans which are glyphosate-resistant and produce Bt toxins. Insects feeding on these plants were more fertile and had significantly more offspring, but both effects were absent if the transgenic soybeans were only resistant to glyphosate. Increasing numbers of white flies also promote plant diseases: the white flies transmit plant viruses when they feed on the plants and their excretions facilitate the occurrence of fungal diseases. Almeida et al. [101] state that the exact causes for the strongly increased number of white flies are unknown. One possibility: the Bt insecticides, which are not toxic for the white flies, might have stimulating effects. Unexpected genomic interactions in the soybean varieties are also being considered. There have been previous reports of an increase in the spread of pest insects in Brazilian transgenic soybean: experts warned in 2014 that another pest insect, the southern armyworm (*Spodoptera eridania*), was increasingly spreading in fields with glyphosate-resistant transgenic soybeans which produce Bt toxins [102]. This effect was apparently not observed in fields with transgenic plants only inheriting one of the traits.

Interactions between the transgenic traits were also described in another case in Brazil [103]: according to their findings, the spread of *Amaranthus* weeds (*Amaranthus palmeri*) in soybean fields is facilitated by the cultivation of the transgenic herbicide-resistant plants. It was found that if black armyworm larvae (*Spodoptera cosmioides*) fed on *A. palmeri*, this had positive effects on its biological and reproductive parameters. In addition, and unexpectedly, the larvae of *S. cosmioides* also show increased fitness if they feed on transgenic soybeans producing Bt toxins (Cry1Ac). Thus, the spread of the larvae benefited from both traits, the herbicide resistance (and its indirect effects) and the Bt trait (and its unintended direct effects). Consequently, the black armyworm, in particular, can become a plant pest in fields where transgenic soybeans with a combination of traits are grown.

There are also some countries in where transgenic organisms from different domains of life were (intentionally or unintentionally) released into an environment

where they might interact with each other. For example, in Brazil, genetically engineered crops [104], fish [105], mosquitoes [106] and pest insects [107] might share the same environment and have some potential to persist and propagate. However, no findings on interactions between those populations have been published.

There are also some findings from transgenic plants which do not include interactions between GE organisms but are, nevertheless, of relevance to the scenarios described above: transgenic wheat with intended resistance to fungal diseases was successfully grown in the greenhouse but showed lower yield and susceptibility to a toxic fungal disease (ergot disease) in field trials [108]. This example shows that the unintended effects of GE organisms may indeed exceed those which can be expected in conventionally bred varieties with the same genetic background. It also shows that environmental conditions may be decisive for the occurrence of unintended effects, which may then also influence interactions between different GE traits within the same species.

It seems that some of this already existing experience may also be relevant in the elaboration of our suggested scenarios. However, due to the specific characteristics of the NGTs summarized above, these scenarios will need further consideration and elaboration before conclusions can be drawn.

Discussion: the need for risk assessment of interactions between NGT organisms and the role of technology assessment

As shown above, the effects resulting from the processes used to generate NGT organisms cannot be generally equated with those derived from conventional breeding, even if no additional genes are inserted. While physical and chemical mutagens (which to some degree also exist in nature) are not likely to cause other mutations compared to those occurring spontaneously, SDN-1 applications of targeted biotechnological mutagens, such as CRISPR/Cas, can produce results (genotypes and phenotypes) that are unlikely to occur with other methods and processes.

If NGT organisms are introduced into the environment on a large-scale, in terms of numbers, traits and events, then such large-scale releases would unavoidably increase the likelihood of direct or indirect effects occurring through interactions between those organisms. Therefore, their intended and unintended interactions and potentially resulting hazards will need specific attention. A lack of awareness of the overall impact of such large-scale releases may have serious consequences for future generations. In the past, technologies meant to solve problems in certain areas (energy, food production, transport) frequently created new problems, e.g., climate

change, nuclear waste, chemical pollution and extinction of species [109]. Given the high technical potential of the above-described NGTs, comprehensive risk assessment may become vital for averting another man-made technology crisis and safeguarding planetary health [110].

NGT organisms which have not adapted through evolutionary processes, may disturb or disrupt ecological networks in many ways. In general, intended or unintended, direct and indirect effects resulting from NGT applications and the subsequent release of the derived organisms, cannot not be seen as 'neutral' to the functioning and services of the ecosystems. From our findings, we identified several factors which are relevant in this context: (1) The differences (in regard to intended and unintended effects) of the NGT organisms compared to plants derived from conventional breeding or found in nature; (2) their combination (caused by simultaneous presence in a shared receiving environment and/or further crossings and/or or technical stacking); and (3) the scale of their release into the environment, which determines exposure and the statistical likelihood of (undesirable) interactions with each other and the environment. Whatever the case, even if the individual events are considered to be safe, the overall scale of releases (number of organisms, different traits and involved species) still needs to be taken into account.

Decision-making on potential releases of NGT organisms in the EU is guided by the precautionary principle in order to prevent eco- and food systems from being confronted with too many risks, uncertainties and unknowns. To this end, the development of hypotheses to explore potential interactions in regard to risk assessment is a crucial step. There are several potential environmental risks that involve interactions between the NGT organisms and the environment, such as (i) disturbance or disruption of ecological interactions of the plants with their associated microbiomes and/or pollinators; (ii) the weakening of resistance (resilience) to biotic or abiotic stressors; (iii) evolutionary mismatch effects that complicate future evolutionary processes; and (iv) endangering biodiversity by the uncontrolled (invasive) spread of NGT organisms and their offspring. Some of these effects have already been shown to be relevant factors in the risk assessment of individual NGT organisms, e.g., *Camelina sativa* with changed oil composition [38]. At the same time, the NGT camelina has the potential to persist in the environment and spread uncontrollably. If these plants are, for example, grown together with other NGT camelina, interactions may occur due to gene flow, crossings and spontaneous offspring between the events. In summary, the hypothesis that the overall impact of several NGT organisms may exceed the sum of effects observed in individual events if they are released

into a shared receiving environment is plausible, especially if this involves large numbers of organisms as well as several traits and/or many species. There may also be potential tipping points linked to parallel or successive large-scale releases of NGT organisms into a shared receiving environment that could trigger irreversible damage.

Interactions between the NGT organisms and other GE organisms may play a decisive role in their overall environmental impact. In this context, it is not only the long-term accumulated effects that should be taken into account, as large numbers and a great variety of NGT organisms may be released into the ecosystems within short periods of time, and thus also trigger unintended combinatorial effects in a short time. These hazards may, however, escape the attention of risk assessors as EFSA [4] guidance does not consider such hazards in detail. Risk assessors may, therefore, see the need to further develop guidelines to ensure that problem formulation and all subsequent steps in risk assessment include all relevant hazards and also leave sufficient space to develop and assess the hypotheses. As Eckerstorfer et al. [111] state, trait related as well as process related effects have to be taken into account in this context: *"To this end, we suggest that two sets of considerations are considered: (1) trait related-considerations to assess the effects associated with the newly developed trait(s); and (2) method-related considerations to assess unintended changes associated with the intended trait(s) or with other modifications in the GE plant (...) Based on these considerations, further guidance should be developed to ensure the high safety standards provided by the current regulatory framework for GMOs in the EU for GE plants in an adequate and efficient way, taking into account the existing knowledge and experience in a case-specific manner. This guidance should thus strengthen the case-specific approach that is recommended by numerous EU and Member States institutions."* Adequate methods to generate the necessary data for risk assessment are, for example, whole genome sequencing, the application of 'omics' [16] and comparative analysis of plant composition and phenotypical characteristics [4]. In addition, potential interactions may be tested in controlled environments [46] and NGT plants can be exposed to defined stress conditions and subjected to experimental crossings [56].

However, even if adequate methodology is applied, it is self-evident that uncertainties will still play a crucial role in this context. As EFSA in its guidance for environmental risk assessment (ERA) correctly states [4, point 2.3.3.8]: *"It is recognised that an ERA is only as good as our state of scientific knowledge at the time it was conducted. Thus, under current EU legislation, ERAs are required to identify areas of uncertainty or risk which*

relate to areas outside current knowledge and the limited scope of the ERA. (...) It is recognised that an environmental risk assessment is limited by the nature, scale and location of experimental releases, which biospheres have been studied and the length of time the studies were conducted.” EFSA [4] proposes several strategies to deal with the different levels of uncertainties and knowledge gaps. However, given the complex nature of effects emerging from interactions between GE organisms, the uncertainties and knowledge gaps might, in many cases, make it impossible to draw sufficiently reliable conclusions. Therefore, cut-off criteria [56, 112] may be necessary to avoid the release of GE organisms (including NGT organisms) if safety is not demonstrated.

In addition, risk assessors and risk managers should consider more general aspects: as argued above, the release of NGT organisms may not be generally considered to be ‘neutral’ to the ecosystems from the perspective of environmental protection. Their (undesirable) impacts may be escalated depending on the number of organisms and traits being released into a shared ecosystem. Similarly to environmental pollution with plastics and chemicals, it is not always an individual product which creates the real problems, but rather the sum of diverse effects. Environmental problems created by the release of NGT organisms may last as long as or longer than those caused by plastics and pesticides, especially if they can persist and propagate in the environment and thus impact many future generations. For this reason, there may be a case for generally restricting the introduction of GE organisms into the environment. Consequently, reliable criteria and robust mechanisms may be needed to justify any releases of these organisms. For example, the EU Commission [75] does discuss the potential benefits of NGT organisms, including mitigating the impacts of climate change or promoting sustainability in agriculture and food production. Therefore, instruments and criteria may be needed to distinguish traits with ‘real benefits’ from those which are simply ‘empty promises’.

On this basis, the elaboration of future scenarios within a prospective technology assessment (TA), in addition to risk assessment, may help to develop regulatory instruments and define criteria to minimize potential adverse effects, and also identify applications with a sound expectation of real benefits. Crucial TA principles are, for example, summarized in the GAO “Technology Assessment Design Handbook” published in 2021: “*New technologies can have a range of effects, potentially both positive and disruptive, that TAs can explore. GAO has broadly defined TA as the thorough and balanced analysis of significant primary, secondary, indirect, and delayed interactions of a technological innovation with society, the*

environment, and the economy and the present and foreseen consequences and effects of those interactions.” [113].

In general, case-specific risk assessment as foreseen in EU GMO regulation can be seen as an ‘end-of-pipe’ safety control mechanism for individual organisms (‘events’) just before they enter the market. In contrast, TA can deal more generally with (groups of) products derived from specific (new) technologies (ideally) before the final products reach the market. In the EU, it could become a tool for making risk management decisions, for example, to control the overall scale of releases of NGT organisms. However, so far, there is no established decision-making process to derive such conclusions. At the same time, the development of suitable methods and criteria is urgently needed: if, in the near future, NGT organisms are released and marketed to the extent proposed by several stakeholders, then interactions between NGT organisms are likely to become unavoidable in many regions of the world, including the EU.

While TA cannot replace the risk assessment of individual organisms (‘events’), it can nevertheless help in political decision-making to seek a balance between the potential benefits and reducing the overall risks of adverse effects on biodiversity and planetary health. If NGT organisms are, for example, applied in agriculture, their potential negative impacts may be minimized by only approving organisms which are considered to be safe and come with a reasonable expectation of providing substantial benefits. Limiting the number and scale of releases of NGT organisms also seems to be a necessary precondition for a reliable assessment of their interactions and associated risks.

Conclusions

New genomic techniques (NGTs) allow new genotypes and traits to be developed in different ways, and with different outcomes compared to previous genetic engineering methods or conventional breeding (including non-targeted mutagenesis).

Plants developed with NGTs may comprise many more different traits and species compared to transgenic plants approved for the market so far. In addition, NGT applications may be relevant to all domains of life, including non-domesticated species.

Due to the intended or unintended effects resulting from the application of NGTs, the release of the NGT organisms may not be ‘neutral’ to the ecosystems. The occurrence of disadvantageous interactions and disruptive effects will be more likely or less likely, depending on how their biological characteristics (intended or unintended) differ from those of conventionally bred or naturally occurring plants, also including the degree of environmental exposure, their combinations and

conditions in the receiving environment. Large-scale releases of NGT organisms across a broad range of species will increase the likelihood of direct and indirect risks associated with interactions between GE organisms, especially in terms of numbers and traits. Whatever the case, it has to be assumed that the overall impact of numerous NGT organisms being released into a shared receiving environment may exceed the sum of effects observed in distinct events. These observations could be a particular cause for concern if NGT-GMOs are released without sufficient regulation and control.

Environmental risk assessment of the individual events is not sufficient to predict or assess these interactions. Consequently, risk assessors and risk managers should not only consider the risks associated with individual NGT organisms, but should also take into account risks emerging from interactions between GE organisms and their potential pathways to harm. Risk scenarios involving several events with different traits, belonging to either the same species or to several species, can be used to develop plausible hypotheses, define hazards and guide the risk assessment in accordance with the precautionary principle. In this context, the sufficiency of the current guidelines for risk assessment should be reviewed in detail and amended as necessary.

Combinatorial effects typically increase the level of complexity and decrease the level of predictability. Therefore, if the complexity of direct and indirect effects caused by interactions in between NGT organisms and with their environment does not allow final conclusions to be drawn on their environmental safety, we propose the introduction of cut-off criteria which allow decisions to be taken in the face of uncertainties [56, 112].

In addition, the risk manager should also consider broader aspects: similarly to the need to reduce the use of plastics and toxic substances, such as pesticides, there may also be a need to restrict the introduction of genetically engineered organisms into the environment. Therefore, reliable instruments and criteria may also be needed to assess the anticipated benefits ensuing from the release of NGT organisms, e.g., mitigating the impact of climate change or promoting sustainability in agriculture and food production. A general limit to the scale of releases of NGT organisms is also likely to be a necessary precondition for a reliable assessment of their interactions. Therefore, additional instruments and criteria established within prospective technology assessment will be needed to distinguish traits with ‘real benefits’ from those which are simply ‘empty’ promises.

Abbreviations

Bt	<i>Bacillus thuringiensis</i>
CRISPR/Cas	Clustered regularly interspaced short palindromic repeats/CRISPR associated
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
EGT	Established genomic techniques involving non-targeted methods for gene transfer
ERA	Environmental risk assessment
EU	European Union
GAO	United States Government Accountability Office
GE	Genetically engineered
GMO	Genetically modified organism
HEGAA	Horizontal Environmental Genetic Alteration Agents
HDR	Homology directed repair
NGT	New genomic technique
NHEJ	Non-homologous end joining
PUFAs	Polyunsaturated fatty acids
SDN	Site directed nuclease
TA	Technology assessment
TALENs	Transcription activator-like effector nucleases

Acknowledgements

We like to thank Nina Valenzuela (Testbiotech) for her editing and the experts of the Bundesamt für Naturschutz (BfN) for giving their feedback.

Author contributions

All authors contributed equally to this manuscript. All authors read and approved the final manuscript.

Funding

Funding was received by the Bundesamt für Naturschutz (BfN), FKZ 3519 84 0300, for those authors (Franziska Koller, Meike Schulz) which are affiliated to the Fachstelle Gentechnik und Umwelt (FGU). Testbiotech was involved without specific funding for this publication.

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 8 October 2022 Accepted: 9 April 2023

Published online: 20 April 2023

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